Hepatology Publish Ahead of Print
DOI:10.1097/HEP.0000000000001160

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Reprogramming macrophages to treat liver diseases

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Word Count Excluding References: 8.065 (including abstract, tables and figure legends, and acknowledgements)

Keywords: Macrophages; innate immune system; liver diseases; modulation

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Conflicts of interest: None declared.

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Abstract

Cutting-edge research has expanded our understanding of the macrophage activation programs in liver diseases making this immune cell type a therapeutic target. Clinical data on macrophage infiltration and polarization states have been used to help predict mortality or poor prognosis in patients with liver cirrhosis and/or hepatocellular carcinoma. The latest single-cell and spatial transcriptomics studies have dissected unforeseen aspects depicting the immense heterogeneity of macrophages and their multifaceted role on both promoting and resolving hepatic inflammation, injury, and fibrosis. Hepatic macrophages (resident tissue Kupffer cells and monocyte-derived macrophages) display such plasticity and phenotypic diversity that macrophages with antagonistic functions may coexist in adjacent regions of the liver. In this scenario, the analysis of macrophage-derived inflammatory and anti-inflammatory circulating soluble markers in patients with liver disease only offers a partial picture of the full complexity of the hepatic macrophage subsets. The reprogramming of macrophages involves understanding the multiple regulatory mechanisms and diverse populations of hepatic macrophages and the design of macrophage-targeted therapeutic interventions to restore hepatic homeostasis. Here we review the potential targets to modulate macrophage behavior in liver diseases and nanoscale therapeutics that aim to target and treat macrophages. We will summarize current knowledge on the diverse macrophage programs activated in chronic liver inflammation, cirrhosis and hepatocellular carcinoma that may be of therapeutic interest for precision medicine.

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1. Introduction

The liver is a key immunological organ ideally positioned for the clearance of diverse antigens and microbial products from the intestinal or arterial blood flow through the action of hepatic phagocytes. Indeed, the liver accommodates the largest number of phagocytes among all the solid organs in the body (1). The hepatic mononuclear phagocyte system comprises monocytes, dendritic cells, and macrophages. Liver macrophages include resident tissue Kupffer cells (KCs) and monocyte-derived macrophages (MDMs) that are rapidly supplemented by large numbers of infiltrating MDMs after liver injury (1). Resident and non-resident liver macrophages display critical but also antagonistic functions by either inducing inflammation, fibrogenesis, and carcinogenesis or promoting anti-inflammatory effects, fibrosis resolution, or anti-tumor immune response (2-4). The relative contributions of these assorted facets of macrophage heterogeneity in controlling specific functions are beginning to be untangled. The use of single-cell and spatial transcriptomics is allowing the study of liver cell compartments, physiology, and disease in unprecedented detail.

The traditional classification of M1 inflammatory and M2 anti-inflammatory macrophages has inevitably been replaced by a model where a myriad of macrophage subsets may spatially and temporally coexist and switch between phenotypes during all stages of liver disease unveiling a very plastic adaptation to the microenvironment. The reprogramming of such cells with pharmacological agents appears to be extremely complex as many different macrophage populations, signals and signaling cascades (e.g. interleukin (IL), toll-like receptors (TLRs), interferon (IFN), or Fcγ receptors) are involved and may deliver multiple downstream responses in the hepatic milieu. The identification of the major regulators that completely control macrophage polarization and the balance between inflammatory and anti-inflammatory signals is important to modulate the role of macrophages in liver diseases (5). Although it is generally considered that macrophage anti-inflammatory pathways favor fibrosis resolution and that pro-inflammatory induction on tumor-associated macrophages help to phagocytose and destroy cancer cells, this is not always necessarily true, and contextualization is required within the complex liver microenvironment. As technology advances, we can now consider the real potential to specifically modulate a precise macrophage population or signaling pathway in liver inflammation, fibrosis, and cancer using therapies bioengineered at the nanoscale (6). The use of

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these macrophage-targeted nanomedicines can help to give insight into the mechanistic effects of modulating specific macrophage subsets in liver disease (7).

This review discusses some of the recent findings on macrophage regulatory programs and surface markers with special emphasis on their heterogeneity and plasticity in regulating liver health and disease. We also introduce the potential of engineered nanoparticles to target and reprogram macrophage subpopulations to study pathophysiological impacts of macrophage modulation in liver diseases.

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2. Hepatic macrophage subsets in health

Hepatic macrophages constitute one of the largest cellular components in the liver and display crucial functions in maintaining liver homeostasis. Hepatic macrophages comprise self-maintaining resident KCs and MDMs. KCs, accounting for 80-90% of liver macrophages (8), originate from colony-stimulating factor 1 receptor positive ($CSF1R^+$) erythromyeloid progenitors (9). KCs are primarily located in the liver sinusoids, extending into the space of Disse, where they interact with hepatic stellate cells (HSCs) and hepatocytes (10, 11). KCs are essential for immune surveillance, pathogen clearance, leukocyte recruitment, and antigen presentation. In mice, KCs express surface markers such as $F4/80^+$, $CD11b^{+/low}$, $CD68^+$, $CD45^+$, and C-type lectin domain family 4 member F ($CLEC4F^+$) along with multiple Toll-like receptors (TLRs) (e.g. TLR4 and TLR9), scavenger receptors and complement receptors (1, 12). KCs maintain liver tolerance by inducing regulatory T (T_{reg}) cells. Unlike MDMs, they are negative for CX3C-chemokine receptor 1 ($CX3CR1^-$) although they share other typical phagocyte surface markers (13). In humans, KCs are commonly identified by $CD14^+$, $CD16^+$, $CD68^+$, $CD163^+$, $MARCO^+$, $CD5L^+$, and $TIMD4^+$ (14, 15). Spatial genomics have revealed other macrophage populations in the healthy liver surrounding the portal triad and the central vein that include: (1) liver capsular macrophages (LCMs), which express general macrophage markers, such as F4/80, CD64, CX3CR1 and CD207 (16, 17); (2) bile duct lipid-associated macrophages (LAMs), which express GPNMB, SPP1, TREM2, CD63, and CD9 (18); and (3) central vein-associated macrophages (CVMs), which express CD207, CCR2, and Chil3 (19).

KCs, strategically positioned in the sinusoids, act as the first line of hepatic defense against circulating pathogens. KCs are decorated with pattern recognition receptors (PRRs) to identify

and clear both exogenous and endogenous particles, such as pathogens, dead cell debris, and toxins. Their strong phagocytic activity is crucial for eliminating gut bacteria before they enter the systemic circulation (20). KCs recognize lipoteichoic acid (LTA) and peptidoglycan in Gram-positive bacteria such as *Staphylococcus aureus* through receptors such as CRIg (complement receptor of the immunoglobulin superfamily) triggering phagocytosis and the release of pro-inflammatory cytokines to recruit additional immune cells (21). KCs detect lipopolysaccharide (LPS) in Gram-negative bacteria through TLR4 (8), which activates nuclear factor kappa B (NF-kB), leading to bacterial phagocytosis and a strong inflammatory response (22). However, these antimicrobial defense mechanisms are significantly impaired in patients with advanced liver disease thus increasing the risk of bacterial infections that are associated with adverse clinical outcomes and mortality (23).

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3. Macrophage heterogeneity in liver diseases

3.1. Macrophage landscape in acute and chronic liver disease

Single-cell studies have revealed that liver macrophages exhibit significant heterogeneity in their surface markers and functions (**Table 1**) depending on their localization in the liver and the disease stage displaying distinct roles in acute and chronic liver injury (2). KCs maintain homeostasis and react to pathogens whereas MDMs infiltrate the liver during injury and contribute to inflammation and fibrosis. This interplay is crucial for understanding liver disease pathogenesis and developing targeted therapies (24).

3.1.1. Macrophages in acute liver injury

Acute liver failure occurs in patients without preexisting liver disease and requires liver transplantation due to the extensive hepatocyte damage and compromised liver function. It is commonly caused by hepatotoxic drugs (e.g. acetaminophen), toxins, or viral infections (e.g. hepatitis) (39). Massive oxidative stress promotes hepatocyte necrosis and the release of damage-

associated molecular patterns (DAMPs), which activate KCs and HSCs. Activated KCs release cytokines and chemokines such as CXC-chemokine ligand 1 (CXCL1), CXCL2 and CXCL8, that recruit neutrophils, as well as CC-chemokine ligand 1 (CCL1), CCL2, CCL25 and CX3CL1, that promote monocyte infiltration (40, 41). Infiltrated LY6C$^+$ monocytes differentiate into MDMs. Initially, LY6C$^+$ MDMs worsen liver injury by releasing pro-inflammatory cytokines (e.g. tumor necrosis factor (TNF), and interleukin-1β (IL-1β)) but then they transition to an anti-inflammatory and resolutive phenotype (LY6C$^-$ MDMs) (1). Resolutive macrophages promote blood vessel restoration by producing vascular endothelial growth factor A (VEGFA) and help restore tissue integrity through the phagocytosis of cell debris and the restructuring of the extracellular matrix (20, 42).

3.1.2. Macrophages in metabolic dysfunction-associated steatotic liver disease (MASLD) and alcoholic liver disease (ALD)

Chronic liver injury occurring in metabolic dysfunction-associated steatotic liver disease (MASLD) or alcoholic liver disease (ALD) may lead to hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (43). MASLD is marked by lipid accumulation in hepatocytes and may progress to metabolic dysfunction-associated steatohepatitis (MASH). Metabolic, environmental and genetic factors contribute to the development of MASLD. Steatotic hepatocytes in MASLD release DAMPs, TNF-related apoptosis-inducing ligand (TRAIL) containing extracellular vesicles (EVs), and free fatty acids (FFA), which activate macrophages though TLR4, inducing the release of pro-inflammatory mediators (e.g. TNF-α and IL-6) that promote disease progression and perpetuate inflammation (44, 45). MASLD may progress to MASH, which is characterized by lobular inflammation, hepatocyte ballooning, severe steatosis, and fibrosis (46). Macrophages play a fundamental role in MASH development as they modulate both hepatic lipid metabolism and inflammation (47).

RNA sequencing studies have evaluated how macrophage subsets are distributed and adapt to changes in MASLD and MASH (19). MASH-associated macrophages express triggering receptor expressed on myeloid cells 2 (TREM2) (48) and are also present in obesity, cirrhosis, and cholestatic liver disease. Two major macrophage subsets with different characteristics but similar roles have been described during the progression from MASLD to MASH (27, 49). The first macrophage subset, SPP1$^+$TREM2$^+$FBP1$^+$GPNMB$^+$ lipid-associated macrophages (LAMs),

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has been found adjacent to fibrotic and inflamed areas (28, 29). Some investigations have revealed novel LAMs anti-inflammatory properties during disease progression since they regulate lipid metabolism and remove cellular debris (30). Amongst LAMs, hepatic crown-shaped structures (hCLS), $CD169^{hi}$ $F4/80^{hi}$ (32), $CX3CR1^{+}$ and $CCR2^{+}$ (50) emerge as a hallmark during MASH progression in mice and humans (51, 52). These cells surround dead or dying hepatocytes and activated HSCs, encircle lipid droplets, and colocalize with collagen fibers. CD11c and CCR2 have proven to be essential for hCLS, since the depletion or deficiency of these markers suppresses hCLS formation in mice (32, 50). The second major macrophage subset during the progression from MASLD to MASH is scar-associated macrophages (SAMs) that are identified as $TREM2^{+}$ and $CD9^{+}$. They are enriched in the fibrotic niche, expand with the onset of liver disease progression, and correlate with therapy outcomes (14, 48, 49, 53).

Alcohol consumption increases gut permeability to bacteria, which leads to higher lipopolysaccharide (LPS) concentration into the portal blood flow. LPS activates KCs through TLR4 and NF-κB signaling triggering hepatic inflammation (54). Ethanol metabolites stimulate the release of reactive oxygen species (ROS) and pro-inflammatory mediators, which exacerbate liver damage and promote steatohepatitis and fibrosis (18). $CCR2^{+}CX3CR1^{+}LY6C^{+}$ monocytes recruited via the CCR2–CCL2 axis infiltrate the liver and give rise to various MDMs populations (KC-like, inflammatory macrophages, and LAMs) that expand the pro-inflammatory reaction (1). However, KCs may also promote anti-inflammatory responses by secreting IL-10 and inducing the apoptosis of pro-inflammatory macrophages in MASLD and ALD (55).

3.1.3. Macrophages in viral hepatitis

Macrophages are key mediators of immune responses in viral hepatitis. KCs detect viral components through their PRRs and TLRs, specifically TLR3, TLR7, and TLR9 (56, 57). These receptors recognize viral RNA or DNA and activate signaling pathways such as the NF-κB and interferon regulatory factor (IRF) cascades, which induce the production of interferons and pro-inflammatory cytokines. Pro-inflammatory monocytes are recruited to the liver after infection with the hepatitis B virus (HBV) or hepatitis C virus (HCV), eventually outnumbering the KCs. Both KCs and MDMs recognize viral components and release inflammatory cytokines such as IL-1β, TNF-α, IL-6, and IFN-α, as well as ROS, FAS ligand (FASL), granzyme B and TRAIL, which inhibit viral replication and recruit additional immune cells (58). Hepatic macrophages

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also aim to contain the infection by releasing pro-fibrogenic factors such as platelet-derived growth factor B (PDGF-BB), TGF-β and galectin 3, which activate HSCs, thus boosting liver fibrosis (58, 59).

3.1.4. Macrophage landscape in liver fibrosis

Macrophages are crucial in the development, progression, and resolution of fibrosis (60). Single-cell RNA sequencing (RNA-seq) clustering has revealed different macrophage subsets in fibrotic livers that may orchestrate either the progression or regression of liver fibrosis (**Figure 1**).

Circulating monocytes can differentiate into pro-fibrogenic $TREM2^{+}CD9^{+}$ SAMs during the progression of liver fibrosis (14). Tissue staining revealed the presence of $TREM2^{+}CD9^{+}MNDA^{+}$ SAMs in collagen positive regions from where they significantly expand in liver cirrhosis. Clusters of KCs are enriched in the KCs expressing CD136, MARCO and TIMD4, but they show a loss of $MARCO^{+}$ cells and a reduction in the number of $MARCO^{+}TIMD4^{-}$ KCs in fibrosis. Self-organizing maps have identified SAM-defining gene signatures, which include TREM2, IL-1β, SPP1, LGALS3, CCR2 and TNFSF12. Some of these genes are responsible for regulating scar-associated myofibroblasts during fibrosis. $TREM2^{+}CD9^{+}$ SAMs present a monocyte-like morphology and a distinct distribution from that of KCs. RNA velocity analysis has shown a downregulation of the monocyte gene MNDA and the lack of KC gene TIMD4 in SAMs, suggesting that SAMs are monocyte-derived cells and represent a differentiated cell type in the fibrotic niche (14, 61). The SAM phenotype has been further characterized by analyzing the monocyte differentiation genes SPP1, LGALS3, CCL2, CXCL8, PDGFB, and VEGF-A, which are also associated with the promotion of tissue fibrosis and angiogenesis (14).

The recruitment of both circulating $LY6C^{hi}$ monocytes and SAMs contributes to liver fibrosis. While both the CCL2/CCR2 and CCL1/CCR8 axis are important for the recruitment of circulating $LY6C^{hi}$ monocytes, macrophage migration inhibitory factor (MIF) contributes to the recruitment of SAMs (62). Chronic liver inflammation perpetuates the fibrotic response through a sustained activation of the HSCs and immune system.

3.1.5. Macrophage landscape in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the two primary malignancies of the liver. HCC commonly arises in patients with underlying fibrosis (63) and is driven by inflammatory cytokines such as TNF-α and IL-6, which activate NF-κB, c-Jun N-terminal kinase (JNK), and signal transducer and activator of transcription 3 (STAT3) (4). Macrophages display dual functions in HCC that may either contribute to tumor progression during chronic inflammation or the clearance of premalignant hepatocytes to prevent HCC (1). Single-cell RNA-seq technologies have been used to characterize and elucidate the immune microenvironment of HCC (64). The tumor microenvironment (TME) in HCC is characterized by chronic inflammation, hepatocyte death and regeneration, and increased ROS production (65). It is enriched with tumor-associated macrophages (TAMs), which secrete cytokines (e.g., IL-1β, IL-6, and TNF) and chemokines (e.g., CCL2 and CXCL10) that enhance tumor growth through the activation of the NF-κB and JNK pathways and the production of pro-angiogenic factors (e.g., VEGF, PDGF, and TGF-β) (1, 4, 66). RNA-seq studies have shown that TAMs mainly express APOE, C1QA, C1QB and TREM2 (64). However, recent single-cell RNA-seq studies have revealed that certain TAM populations express both M1-like inflammatory and M2-like anti-inflammatory markers, indicating that they represent a highly heterogeneous cell population that can exert either pro-tumoral or anti-tumoral functions (67, 68) (**Figure 2**).

TLR4 and TLR9 are overexpressed in TAMs and their activation in HCC induces downstream IL-6 production through TLR/MyD88 signaling, which promotes tumor cell growth via the STAT3 pathway, a major driver of hepatocyte repair and replication (4, 69). TAMs release anti-inflammatory cytokines that promote cancer cell proliferation, invasion, tumor metastasis, and angiogenesis, as well as actively participate in immune suppression via immune checkpoint molecules such as programmed death ligand 1 (PD-L1) and IL-10 (1). Nonetheless, hepatic macrophages also participate in anti-tumor responses. M1-like inflammatory macrophages possess tumoricidal properties since they release inflammatory cytokines that hinder cellular proliferation and dampen tumor progression (70). These anticancer properties of macrophages in HCC are generating great in the design of immunotherapies that aim to improve the prognosis and quality of life of patients with HCC (71).

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4. Targets to reprogram macrophages in MASLD and MASH

4.1. Activation of macrophages in the context of MASLD and MASH

Progression from MASLD to MASH involves the acquisition of an inflammatory state in the liver parenchyma, for which macrophage activation and recruitment are essential (2) (**Figure 3**). Macrophages can be activated by the molecules released by extrahepatic tissues, which are also involved in the development of metabolic syndrome. In this context, increased intestinal permeabilization contributes to hepatic macrophage activation through the entry of pathogen-associated molecular patterns (PAMPs) and the pro-inflammatory signaling molecules secreted by intestinal macrophages (72, 73). Indeed, cirrhosis-associated dysbiosis has been described to exacerbate the progression of MASH (74). Adipose hyperplasia and adipocyte necrosis leads to FFA shredding, which activates both adipose and liver macrophages. Activated $CCR2^{+}$ adipose tissue macrophages secrete cytokines such as TNF-α and IL-6 (75), which reach the liver macrophages and contribute to their activation. Increased leptin secretion by the adipose tissue (76) leads to the phosphorylation of AKT and ERK1/2 in KCs, subsequently increasing NF-κB signaling and the expression of TGF-β1 and CTGF/CCN2 (77). Hepatocyte EVs loaded with TRAIL regulate NF-κB and DR5 activity in bone marrow-derived macrophages (BMDMs) promoting the release of IL-1β and IL-6 (77). Hepatocytes may also release mtDNA and contribute to hepatic macrophage activation through TLR9 (78).

4.2. Signaling pathways associated with macrophage polarization

Liver macrophages are very plastic cells that can polarize into different subtypes during MASH. C-C chemokine receptor type 1 (CCR1), a G protein-coupled receptor, is one of the molecules that control the phenotype shift towards pro-inflammatory macrophages. CCR1 regulates calcium–calmodulin-dependent protein kinase II (CaMKII), which in turn increases AMP-activated protein kinase (AMPK) expression, activating NF-κB signaling and inhibiting mechanistic target of rapamycin complex 1 (mTORC1). Hence, CCR1 activation increases hepatic oxidative stress and promotes a pro-inflammatory polarization in macrophages (79). Common inflammatory mediators such as, TNF-α, IL-1β and IFN-□ modulate NF-κB signaling in liver macrophages, perpetuating this pro-inflammatory state (47).

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Metabolic reprogramming has been reported to polarize pro-inflammatory macrophages towards a resolutive phenotype (79, 80). Annexin A5 can switch glycolysis towards oxidative phosphorylation, inducing a resolutive phenotype (79). Other studies have shown that celastrol, through the covalent modification of the Cys31 residue of pyruvate kinase M2 (PKM2), can influence AKT-mTOR-HIF1α-PKM2 signaling, thereby promoting a macrophage anti-inflammatory and resolutive phenotype shift in mice (80).

FFAs can play a dual role in liver macrophage polarization promoting either the inflammatory or anti-inflammatory state. Macrophage activation by LPS and FFAs through TLR4 can contribute to both lipid synthesis and accumulation in hepatocytes as well as a shift towards a pro-inflammatory phenotype (47, 79). By contrast, short chain fatty acids (SCFAs) activate peroxisome proliferator-activated receptor gamma (PPARγ) (81), which binds to the MMP-10 promoter to activate the STAT3 pathway culminating in a decrease in IL-1β and TNF-α expression, and an increase in IL-10 levels that stimulate a resolutive phenotype in hepatic macrophages (82). These resolutive macrophages may be defined as those macrophage subsets that exert control over the microenvironment to mitigate the inflammatory response and stimulate remodeling of the ECM for tissue repair to boost homeostasis restoration.

4.3. Drug therapies to reprogram macrophages in MASLD and MASH

To date, only the liver-targeting drug resmetirom has been approved by the US Food and Drug Administration (FDA) for MASH treatment (83) (**Table 2**). This is due, in part, to the standard of care in patients with MASH, which is mainly based on changes in daily lifestyle (diet, changing harmful habits, and exercise) instead of drug therapies (46, 84). Since liver macrophages contribute to lipid metabolism/accumulation, inflammation, and progression from MASLD or MASH to cirrhosis (85, 86), the design of macrophage-specific drugs is emerging as a potential therapeutic strategy for fighting the advance of chronic liver disease. The reprogramming of the macrophage phenotype of LAMs, SAMs or hCLS is still controversial although it has been proposed to reverse the progression of MASH. Lipid active molecules such as 19,20-epoxy docosapentaenoic acid (19,20-EpDPE) have been demonstrated to attenuate hCLS formation via the GPR120 macrophage receptor in a Fat-1 transgenic mouse model (52).

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FGF21 prevents hepatic lipotoxicity and inflammation promoting FFA oxidation in hepatocytes and reducing the expression of pro-inflammatory mediators such as TNF-α, IL-1β and CCL2. Consequently, SAMs, LAMs and hCLS polarization decreases, which correlates with COL1A1 synthesis inhibition (87). Dual agonists for PPARα and PPARγ such as elafibranor and rosiglitazone act at two different levels. First, they promote a phenotypical shift towards an anti-inflammatory macrophage phenotype and second, PPARα upregulation in hepatocytes promotes lipid metabolism and decreases oxidative stress (88), leading to a reduction in lipid synthesis and accumulation in the livers of treated mice (47, 89) (**Table 2**). Unfortunately, these results have not been translated from bench to bedside as PPAR agonists clinical trials have failed despite the promising results demonstrated in preclinical murine models (90). Of note, PPARα in human hepatocytes is under-expressed compared to mice (89), which may partially explain why clinical trials have not been successful so far. In addition, the PPARα and PPARγ inhibitor elafibranor has demonstrated off-target effects upregulating epithelial to mesenchymal transition in hepatocytes (91).

Drugs targeting the inflammatory response driven by macrophages are also currently being developed. In this context, treatment with sodium acetate (NaA) activates the AMPK signaling pathway, reducing the expression of TNF-α and IL-6 via JNK and NF-κB that leads to a decrease in inflammation and macrophage infiltration in a mouse model using a methionine- and choline-deficient (MCD) diet (81). The targeted activation of TIM3 increases the antioxidant capacity of mouse liver macrophages, resulting in decreased ROS production and macrophage activation that exert hepatoprotective effects (84). Low doses of the anti-CD163-IgG-dexamethasone conjugate have been observed to reduce inflammation, hepatocyte ballooning and fibrosis in a rat model via the targeting of CD263 (hemoglobin scavenger receptor) (94). Controlling monocyte and lymphocyte recruitment could also have therapeutic potential for reducing MASH progression. However, Cenicriviroc, a CCR2/CCR5 antagonist that targets macrophage recruitment by CCL2 (105), has shown no efficacy in the treatment of MASH, despite a recent phase III randomized study showing that it was a safe and well tolerated drug (ClinicalTrials.gov, number: NCT03028740) (**Table 2**) (96).

5. Targets to reprogram macrophages in hepatic ECM remodeling

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5.1. Interplay between macrophages and other hepatic cells driving fibrogenesis

The interplay between macrophages and other hepatic cells coordinates fibrogenesis and ECM remodeling. One of the most important crosstalk occurs between macrophages and HSCs. It has been proposed that KCs control liver fibrogenesis through the CCR9/CCL25 axis by activating the expression of α-SMA, TGF-β1, collagen 1α1 and TIMP-1 mRNA in HSCs (106). Macrophages can also activate HSCs through TGF-β, IL-6 and IL-17. Indeed, TGF-β is the most powerful stimulus for the activation of HSCs (107). TGF-β also contributes to the survival of activated HSC by decreasing natural killer antifibrotic activity (108). IL-1 and TNF-α secreted by macrophages may also modulate the survival of activated HSCs by inducing the NF-kB pathway, leading to B cell lymphoma 2 (BCL-2) upregulation, and the inhibition of the expression of Bax, PUMA and p53 (109, 110). Contrarily, MMP-9 and TRAIL expression by macrophages may cause HSC apoptosis(103). Moreover, IL-22 downregulates the ERK1/2 and AKT pathways and simultaneously activate STAT3 signaling in macrophages, which results in HSC deactivation and collagen expression inhibition and promotion of hepatocyte survival (111). Hence, macrophage secretome modulates both HSC survival and apoptosis. Indeed, $LY6^{Chi}$ macrophages have been described to sustain HSC activation, but a high M2/M1-KCs ratio has been proposed to inhibit collagen production and decrease HSC activation (111). HSCs may also influence macrophage resolutive functions by secreting TIMP-1, which inhibits MMP-13 expression (112).

The interplay of macrophages with other immune cells also affects the progression of liver fibrosis. $LY6C^{hi}$ monocytes/macrophages modulate neutrophil activation during the pro-inflammatory phase and can also contribute to neutrophil apoptosis during the resolution phase (113). Neutrophils, in turn, control IL-10 and IL-12 macrophage expression via miR-223 EVs, stimulating resolutive phenotype polarization in macrophages (114). Mucosal-associated invariant T (MAIT) cell activation promotes fibrogenesis via the stimulation of the inflammatory macrophage phenotype through an increase in the CCR2/CD206 ratio (100). Moreover, pro-inflammatory macrophages may, in turn, promote MAIT cell activation via MHC-related protein-1 (MR1) -T cell receptor (TCR) dependent interactions, driven by bacterial ligands presented through MR1 by macrophages, and cytokines, such as IL-12 and IL-18 (100).

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The major influence in macrophage inflammatory profile is exerted by HSCs and immune cells but hepatocytes may also regulate macrophage recruitment via cytokine expression during early stages of fibrosis (41, 46). DAMPs, cytokines and ROS released from injured hepatocytes during fibrosis progression may activate Kupffer cells TNF-α, TGF-β, PDGF-BB and IL-1β release and ROS production (115). Hepatocytes additionally contribute to LY6C^{hi} monocyte recruitment through CCL2 expression, further supporting the increase in liver macrophage pool and endorsing liver inflammation and HSC activation (41).

5.2. Role of macrophages in ECM accumulation, degradation, and fibrosis resolution

Macrophages play an important role as drivers of the fibrogenic response and are also crucial players in the resolution phase by stimulating remodeling of the extracellular matrix. Monocyte recruitment into an injured liver is mediated by the CCR2/CCL2 axis with MMP-13 enabling tissue infiltration. Infiltrated monocytes transdifferentiate into LY6C^{hi} macrophages and secrete TGF-β and PDGF-BB, which promote HSC activation and fibrogenesis. Monocyte recruitment to an injured liver occurs during the first 48 hours. Early on during that timeline, from 12-36 hours, a decrease in LY6C expression (116) can predict the phenotype shift of macrophages towards a resolutive state (101). IL-10, a classical pro-resolutive cytokine, reduces the release of macrophage inflammatory mediators via iNOS and ROS inhibition (117) and promotes angiogenesis and necrotic/apoptotic cell engulfment in combination with TGF-β. Arginase-1 (Arg-1) expression is induced during the resolutive phase by CX3CL1, amongst other cytokines (118). Arg-1 competes with iNOS for L-arginine leading to the apoptosis of pro-inflammatory iNOS-expressing cells (55). Cytokine-independent pathways, such as phagocytosis, contribute to the macrophage resolutive phenotype through the activation of ERK signaling, and matrix degradation via the synthesis of MMPs (62, 101).

ECM remodeling is executed by MMPs, whose inhibitors, TIMPs, are regulated to preserve a homeostatic balance in physiological conditions (119). In homeostasis, extracellular matrix is degraded by MMP-13 and MMP-3, controlled by TIMP-2 (120). The protease balance is dysregulated during the advance of liver fibrosis in chronic liver disease because HSCs express TIMP-1 (120), which forms inactive MMPs complexes (121). The subset of LY6C^{lo}

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resolutive macrophages displays a high expression of the genes associated with ECM remodeling. *In vitro*, it has been demonstrated that the phagocytosis occurring in $LY6C^{lo}$ liver macrophages activates ERK signaling, leading to the activation of MMP-9, MMP-12, insulin-like grow factor (IGF1) and GPNMB expression (101). MMP-13 has been observed to have paradoxical roles in CCl_4-treated mice, not only facilitating the macrophage expression of MMP-2 and MMP-9 (that partially degrade denatured collagen) but also upregulating the mRNA levels of inflammatory and fibrogenic mediators such as TNF-α and TGF-β1 (122). Other studies in CCl_4-treated mice have correlated the expression of MMP-13 with an increased expression of collagenase and MMPs, favoring ECM degradation with SAMs (123).

The continuous accumulation of ECM during fibrogenesis leads to local hepatic hypoxia, which stimulates hypoxia inducible factor 1-α (HIF-1α) and VEGF expression in macrophages. Myeloid VEGF-A restores collagenolytic capacity of macrophages through the upregulation of MMP-2 and MMP-14 expression, the downregulation of TIMP-1 and TIMP-2, and a reduction in the number of myofibroblasts (124, 125).

Lysosomal dysfunction contributes to shift towards a pro-inflammatory/fibrotic macrophage phenotype during liver fibrosis. This dysfunction can be caused by multiple factors, such as cholesterol accumulation due to the engulfment of dead hepatocytes (126), LPS-mediated stimulation of macrophages, or the deregulation of the protease network (127, 128). Since autophagy is one of the key cellular processes controlled by the lysosome, it is not surprising that autophagic fluxes influence ECM remodeling and macrophage polarization (126, 127). Therefore, correct lysosome function is crucial for fibrosis resolution.

5.3. Targeting and treating macrophages in liver fibrosis and cirrhosis

Macrophage polarization and activity during liver fibrosis are regulated by a wide array of signaling pathways including TGF-β/Smad, NF-κB, and STAT3. In hepatic fibrosis, KCs and $LY6C^{+}$ MDMs activate HSCs and support transdifferentiation into myofibroblasts and survival by secreting TGF-β1 and PDGF-BB (2). The inflammatory cytokines released by macrophages such as TNF-α and IL-1β perpetuate the immune reaction, tissue injury, and inflammation. By contrast, $LY6C^{-}$ resolutive MDMs can replace SAMs to facilitate fibrosis resolution (129), since they counteract fibrosis by deactivating HSCs (senescence, apoptosis or reversion to a quiescent

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state) (101). LY6C$^-$ resolutive MDMs also promote tissue repair by dampening inflammation and releasing anti-inflammatory cytokines such as IL-10 (130). (**Figure 4**)

Most therapeutic approaches for liver fibrosis have focused on targeting HSC activation, hepatocyte regeneration or angiogenesis. However, antifibrotic therapeutic strategies that modulate macrophage polarization, inhibit KC activation, and prevent chemokine-mediated monocyte recruitment have shown promising results (24, 41). It is important to emphasize that the timing of the treatment of liver fibrosis through the modulation of macrophages is key since they play different roles throughout the course of the disease. The first approach in reprogramming of macrophages is through the control of their inflammatory profile. Different, even opposite, results have been obtained depending on the therapeutic candidate. Selonsertib, an ASK1 inhibitor, demonstrated no antifibrotic effects and a lack of efficacy for MASH treatment in a phase III trial (NCT03053050 and NCT03053063) (98). However, Lanifibranor, a pan-PPAR agonist, that has been shown to induce an anti-inflammatory polarization shift in macrophages in preclinical models and succeeded in a phase II-b clinical trial with 247 patients (NCT03008070) (99) (**Table 2**). Anti-MR1 antibodies and Ac-6-FP bind to macrophage MR1 and compete with TCR, MAIT activation ligand, further promoting the resolutive phenotype of macrophages. This therapy has been shown to downregulate CCL2 and its receptor CCR2, while upregulating autophagy-related genes such as ATGs and LC3 in preclinical models (100) (**Table 2**).

The activation of phagocytosis by liposome administration has also been investigated in preclinical trials. This strategy promotes fibrosis resolution through a shift towards a resolutive macrophage phenotype in a mouse model of liver fibrosis (101). In 2011, the delivery of BMDMs in a preclinical model showed promising results upregulating the recruitment of MMP13$^+$ macrophages and MMP9$^+$ neutrophils to fibrotic scars (102). Consistent with this finding, a first-in-human phase I clinical trial in 2019 (ISRCTN 10368050) with 9 patients demonstrated that the delivery of autologous macrophages is safe and feasible for the treatment of liver fibrosis (**Table 2**) (104).

Emerging research and development in the field of cell therapy using CAR-M (Chimeric Antigen Receptor Macrophage), similar to CAR-T therapy but using macrophages, has shown promising results (103). These data show genetically engineered bone marrow-derived

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macrophages with a chimeric antigen receptor targeting the senescence marker urokinase plasminogen activated receptor (uPAR) that achieved attenuation of liver fibrosis and cirrhosis in preclinical models. This CAR-M strategy targeted the macrophage phagocytic activity to act against HSCs and triggered a T-cell immune response, leading to fibrosis regression and liver function restoration. This cell-based therapy provides a new strategy to reprogram macrophages to help in the treatment of liver fibrosis and cirrhosis.

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6. Targets to reprogram TAMs in HCC

TAMs in HCC originate from peripheral blood monocytes, myeloid-derived suppressor cells (MDSCs), and KCs (131). TAMs are one of the main tumor-infiltrating immune cell types and are generally considered to acquire an immunosuppressive phenotype due to the influence of adjacent cancer cells (132). Positionally and phenotypically, TAMs have been typically divided into two subgroups: M1-like classically activated TAMs and M2-like alternatively activated TAMs (133). Classical TAMs are mainly present in the tissue adjacent to the tumor, whereas alternative TAMs are mainly present in the liver tumor mass (133). However, discoveries using single-cell sequencing technologies in biopsies from patients with HCC have challenged these conventional concepts (64). Indeed, tumor tissues from patients with HCC exhibit clusters of macrophages enriched with signatures for MDSCs or TAMs and simultaneous signatures of M1-like and M2-like macrophages (64). The co-existence of M1 and M2 signatures indicates that TAMs are more complex than the classical M1/M2 model, consistent with some findings in other tumors such as breast cancer (134). A diffusion map of their global transcriptomes depicts that MDSC-like macrophages highly express the S100A family genes FCN1 (Ficolin 1) and VCAN (Versican), whereas TAM-like cells express genes that are also found in the TAMs of lung cancer (135), including APOE, C1QA, C1QB, and TREM2 (64). Specific transcripts of SLC40A1, encoding ferroportin, and GPNMB (encoding type I transmembrane glycoprotein) are also highly expressed in TAMs. The transcription factors that regulate these two clusters in TAMs include ID3, MITF, RUNX2, and MAF. Indeed, specific gene signatures of SLC40A1 and GPNMB in TAMs are associated with poor prognosis in HCC, emphasizing the importance of these two programs in the regulation of TAMs states in HCC (64).

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TAMs contribute to the stemness of hepatocellular carcinoma cells through the secretion of inflammatory stimuli and the activation of the NF-κB signaling pathway (136). Namely, cytokines (TNF-α, IL-6, and TGF-β) and a reduced presence of microRNAs (miRNAs, such as miR-125a and miR-125b) derived from M2-like TAMs may promote cancer cell stemness and expansion (137, 138). The upregulation of the macrophage-induced long noncoding RNA H19 also enhances stemness and promotes tumorigenesis (139). Indeed, macrophage induced H19 levels significantly correlate with HCC progression and aggressiveness (139). The communication between TAMs and cancer stem cells (CSCs) is very active. For example, CSCs secrete periostin (a member of the fasciclin family), which promotes the recruitment of M2-like TAMs (140) and cytokines such as IL-13, osteoactivin, and IL-34 that transdifferentiate macrophage precursors into the M2-like cancer-promoting phenotype (141) in cholangiocarcinoma. TAMs release CCL17 that may stimulate tumor stemness and epithelial mesenchymal transition through the TGF-β1 and Wnt/β-catenin signaling pathways (142). Therefore, a potential therapeutic approach for liver cancer might be blocking the communication between M2-like TAMs and liver cancer cells using noncoding RNA inhibitors and TAM-related receptor inhibitors.

Tie2-expressing monocytes (TEMs) and TAMs are found in the perivascular area of tumor tissues and actively participate in HCC angiogenesis (71, 143). Indeed, the quantification of infiltrated pro-angiogenic Tie2-expressing TAMs shows some potential as a diagnostic marker for HCC (143). The interplay between TAMs or TEMs and the tumor vascular network, which allow tumor growth and expansion, is in part mediated by pro-angiogenic factors such as VEGF, angiopoietins, PDGF, TGF-β, and FGF, among others (144, 145). The presence of pro-angiogenic TAMs correlates with the appearance of resistance to anti-VEGF therapy (146). This suggests that the combination of VEGF inhibitors with macrophage receptor blockers (such as CSF1 or CCR2 inhibitors) could improve the anti-VEGF therapeutic response (147, 148). Highly vascularized HCC shows high numbers of $CCR2^+$ TAMs at the margins of the tumor (149). The absence of $CCR2^+$ TAM infiltration has been shown to mitigate pathogenic angiogenesis (149). Proangiogenic TAMs combine the release of VEGF with that of inflammatory cytokines to induce pathogenic angiogenesis. A case-control study showed that hepatocytes infected with the hepatitis B virus induced $CD14^+$ inflammatory macrophages to release IL-23 and upregulate IL-23 receptors, which have been associated with an intense stimulation of macrophage-associated

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tumor angiogenesis (150). Hypoxia is one of the stimuli that induce TAM angiogenic and immunosuppressive phenotypes. One example of the programs induced in hypoxic TAMs is the regulated in development and DNA damage response 1 (REDD1), which is a negative regulator of mTOR, the main target of the anticancer drug rapamycin. This inhibitor can hamper glycolysis and the proangiogenic response in TAMs. REDD1-knockout TAMs have been observed to show hindered glycolysis and re-establish abnormal angiogenesis and metastases (151). Metabolic reprogramming may also be involved in the differentiation process from a quiescent to an activated state. Studies in mice and humans have shown that glycolytic metabolism is implicated in the classical activation of M1-like TAMs while mitochondrial oxidative phosphorylation is related to the alternative activation of M2-like TAMs (152). However, macrophage metabolism is more complex than a binary selection. For example, elevated glycolysis can control PD-L1 levels and induce M2-like polarization (153) or, in some specific macrophages displaying an HLA-DR^{high}CD86^{high} phenotype, glycolytic metabolism may confer PD-L1^{+} macrophages with anti-tumorigenic properties in HCC (154). Some HCC-derived factors can also induce glycolysis in M2-like TAMs via Wnt2b/β-catenin/c-Myc signaling (155). Similarly, lipid metabolism in TAMs is also required for macrophage polarization and hepatocarcinogenesis. For example, a reduction in receptor-interacting protein kinase 3 (RIPK3) levels in TAMs decreases ROS levels and induces fatty acid metabolism through PPAR activation, resulting in the accumulation and polarization of M2-like TAMs (156).

Induced autophagy in TAMs leads to macrophage repolarization from the M2-like to the M1-like phenotype followed by secretion of anti-tumor cytokines (157). TAMs can also induce autophagy in HCC cells and attenuate the toxic effects of chemotherapy such as oxaliplatin (158). TAM autophagy and M2 polarization can be triggered by the HCC-derived high-mobility group box 1 (HMGB1)/via a Toll-like receptor 2TLR2/NADPH oxidase 2 (HMGB1/TLR2/NOX2) axis to sustain liver cancer growth (159). Therefore, the modulation of programs controlling autophagy in TAMs could be a promising strategy to impair HCC growth and progression.

7. Nanoparticles to target and reprogram macrophages in liver diseases

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Conventional pharmacological agents to treat liver diseases show limitations due to either intrinsic pharmacokinetic/pharmacodynamic properties or the barriers of hepatic pathophysiology. Moreover, off-target effects of current drugs in a setting of compromised liver metabolic function increases the risks of adverse effects. Even specific monoclonal antibodies used in the treatment of liver cancer have shown adverse reactions (160). In this scenario, a promising avenue to develop new therapies for patients with liver disease involves the modulation of macrophages using targeted nanomedicines (6) (**Figure 5**). The rapid detection and clearance of foreign compounds by the mononuclear phagocyte system leads to a natural accumulation of most nanoparticles in the spleen, lymph nodes, and liver (the main organs of the mononuclear phagocyte system) (161). Namely, nanoparticles commonly accumulate in inflammatory macrophages at diseased sites (7, 162, 163). The chronic inflammation occurring in liver diseases meets the conditions for prolonged macrophage activation (1) and, thus, the uptake of macrophage-targeted nanomedicines.

Different therapies engineered at the nanoscale have recently shown meritorious potential for reprogramming macrophages in chronic liver disease (CLD) (6). Graphite-based nanoparticles linked to poly(amidoamine) dendrimers (DGNPs) have been used to selectively deliver DNA plasmids expressing the ring finger protein 41 (RNF41), an E3 ubiquitin protein ligase, to hepatic $CD11b^+$ MDMs (33). These expression plasmids for RNF41 were designed with a CD11b promoter (to guarantee that only the recruited inflammatory macrophages expressed that protein) and an enhanced green fluorescent protein (EGFP) reporter gene under the control of a strong and ubiquitous promoter (cytomegalovirus). This macrophage-targeted nanomedicine was designed because it was found that RNF41 macrophage mRNA expression was markedly lower in the macrophages from cirrhotic livers than from healthy livers, regardless of the etiology of the cirrhosis (alcoholic, nonalcoholic, hepatitis C, or autoimmune), and was also lower in mice with liver fibrosis induced by i.p. CCl_4 or thioacetamide (TAA). The therapeutic strategy of the nanomedicine aimed to restore RNF41 expression levels exclusively in hepatic MDMs. Indeed, it was demonstrated that the fluorescence signal corresponding to the EGFP reporter was found in $CD11b^+$ macrophages, with negligible signals in hepatocytes, HSCs, and LSECs (33). Restored macrophage RNF41 expression using DGNPs reduced liver inflammation and fibrosis and induced hepatic regeneration in mice with fibrosis and hepatectomy. This treatment also reduced the secretion of pro-fibrogenic TGF-β and PDGF-BB

in hepatic macrophages and increased the expression of MMP-9, suggesting a synchronized crosstalk between macrophages and HSCs. The hepatocyte proliferation induced by the recovery of macrophage RNF41 expression has been attributed to the up-regulation of IGF-1 expression. Moreover, the prolonged inflammatory signal from TNF-α promoted the downregulation of macrophage RNF41 expression due to the continuous and progressive increase in stress-activated MAPK signaling, which subsequently leads to ERK-induced AKT down-regulation (164), and the reciprocal inhibitory effects between RNF41 and ERK (165). Several lines of evidence indicate that AKT leads to M2-like polarization, since AKT deficiency or AKT signaling inhibition, abolishes the up-regulation of the genes associated with the M2 phenotype (166). Indeed, M2 polarization is dependent on PI3K/AKT signaling via TGF-β or IL-10-induction (166). Therefore, the modulation of macrophage AKT appears to be a potential target in controlling macrophage RNF41 expression and the M2 phenotype in CLD.

Dendrimer-graphene nanostars (DGNS) have also been employed for delivering an expression plasmid under a CD11b promoter into liver MDMs (167). This nanomedicine was designed to produce high amounts of the collagen-degrading enzyme MMP-9 in the inflammatory macrophages that accumulate in fibrotic tracts during hepatic fibrosis. This macrophage-targeted gene therapy selectively and locally reduces the presence of collagen fibers in the livers of fibrotic mice leading to a significant decrease in hepatocyte damage (levels of serum transaminases) and improved synthesis of albumin and proteins (167). DGNS have also been attached to other therapeutic compounds, such as the PPARγ agonist GW1929, to investigate the effects of selectively activating this pathway on macrophages in liver inflammation and fibrosis (95). This nanomedicine allowed the selective delivery of GW1929 to macrophages in the mouse fibrotic liver at a dose lower than that previously reported, resulting in a rapid switch to an anti-inflammatory macrophage phenotype (95). The reduction of hepatic inflammation due to macrophage-targeted GW1929-DGNS treatment was associated with a significant reduction in hepatic fibrosis but no improvements in liver function or HSC activation were observed. Therefore, these carbon-based NPs are excellent technologies that can be used to gain insight into the precise mechanisms and signaling pathways involved in macrophage activation and their downstream effects in CLD.

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Lipid NPs have been used for the transport of RNA to different hepatic cells in health and disease (168, 169). These NPs have been adjusted and functionalized to increase RNA delivery to hepatic macrophages for the treatment of chronic inflammation and fibrosis. Some studies have incorporated mannose onto the surface of these NPs to target the mannose receptors on hepatic macrophages and deliver the siRNA against the inflammatory factor HMGB1 in a mouse model of MASH (92). Other studies have used lipid NPs to deliver siRNA to TAMs (93). For example, STAT3 and HIF-1α have been targeted to enhance the level of infiltrated macrophages ($CD11b^+$ cells) in the tumor microenvironment (TME) to induce an anti-tumor response (27).

8. Concluding Remarks and Future Perspectives

The multifaceted contributions of macrophages extend far beyond the phagocytosis of invading pathogens. Macrophages are the gatekeepers of homeostasis and the architects of tissue integrity and remodeling during all stages of CLD. They orchestrate immunological responses against injury and modulate ECM production/degradation via the activation/inhibition of HSCs or ECM-degrading enzymes to either contain the damage or stimulating fibrosis resolution. Macrophages also have a deep impact on regulating the TME in liver cancer. Different regulatory programs and macrophage subsets are involved in the global impact of macrophage functions in liver diseases. The immense plasticity and heterogeneity of liver macrophages in the setting of each etiology of CLD highlights the importance of defining which macrophage subset should be selected as a target to either potentiate or inhibit their activity in precision medicine. Therapies targeting the inflammatory NF-κB or anti-inflammatory PPARs pathways are still the standard for reprogramming macrophages in alcoholic hepatitis, MASLD, and MASH. The promotion of the anti-inflammatory macrophage phenotype also results in a reduced fibrogenesis due to the close relationship between hepatic macrophages and HSCs in liver cirrhosis. By contrast, strategies to treat TAMs in liver cancer aim to stimulate a pro-inflammatory and antiangiogenic phenotype for the detection and destruction of cancer cells.

Advances in nanotechnology in recent years have yielded a diverse array of nanomedicines that selectively modulate macrophage behavior to treat liver inflammation, fibrosis, and cancer. The complexity of the architecture and vascular irrigation of the liver in

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CLD complicates the task of ensuring that the nanomedicine reaches its intended target without affecting other tissues. Nanomedicine is at the forefront of innovative therapies, offering new possibilities for the treatment of CLD and HCC through the modulation of macrophage activity.

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Acknowledgments

This work was supported by grants from the Ministerio de Ciencia, Innovación y Universidades MICIU/AEI/10.13039/501100011033 (PID2021-123426OB-I00 and by "ERDF A way of making Europe"; and CNS2022-135148 and by the "European Union NextGenerationEU/PRTR") to P.M-L. P.M-L. was additionally supported by a fellowship from the Ramon y Cajal Program (RYC2018-0Z23971-I) from the Spanish Ministerio de Ciencia, Innovación y Universidades and by the FSE invierte en tu futuro. A.M. is funded by MCIN/AEI/10.13039/501100011033/, by FEDER a way of making Europe through the project grant PID2021-123652OB-I00 and by Pfizer grant #77131383. B.S-C had a Formación de Profesorado Universitario (FPU) grant from Ministerio de Ciencia, Innovación y Universidades and FSE invierte en tu futuro (reference: FPU22/02178). J.C-P. is funded by MCIN/AEI /10.13039/501100011033 contract PRE2022-101676.

The Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd) is funded by the Instituto de Salud Carlos III. RedFibro (RED2022-134485-T) for aid to «RESEARCH NETWORKS», within the framework of the Programa Estatal del Plan Estatal de Investigación Científica, Técnica y de Innovación 2021–2023. Consolidated Research Group of the Generalitat de Catalunya AGAUR (2021 SGR 00881).

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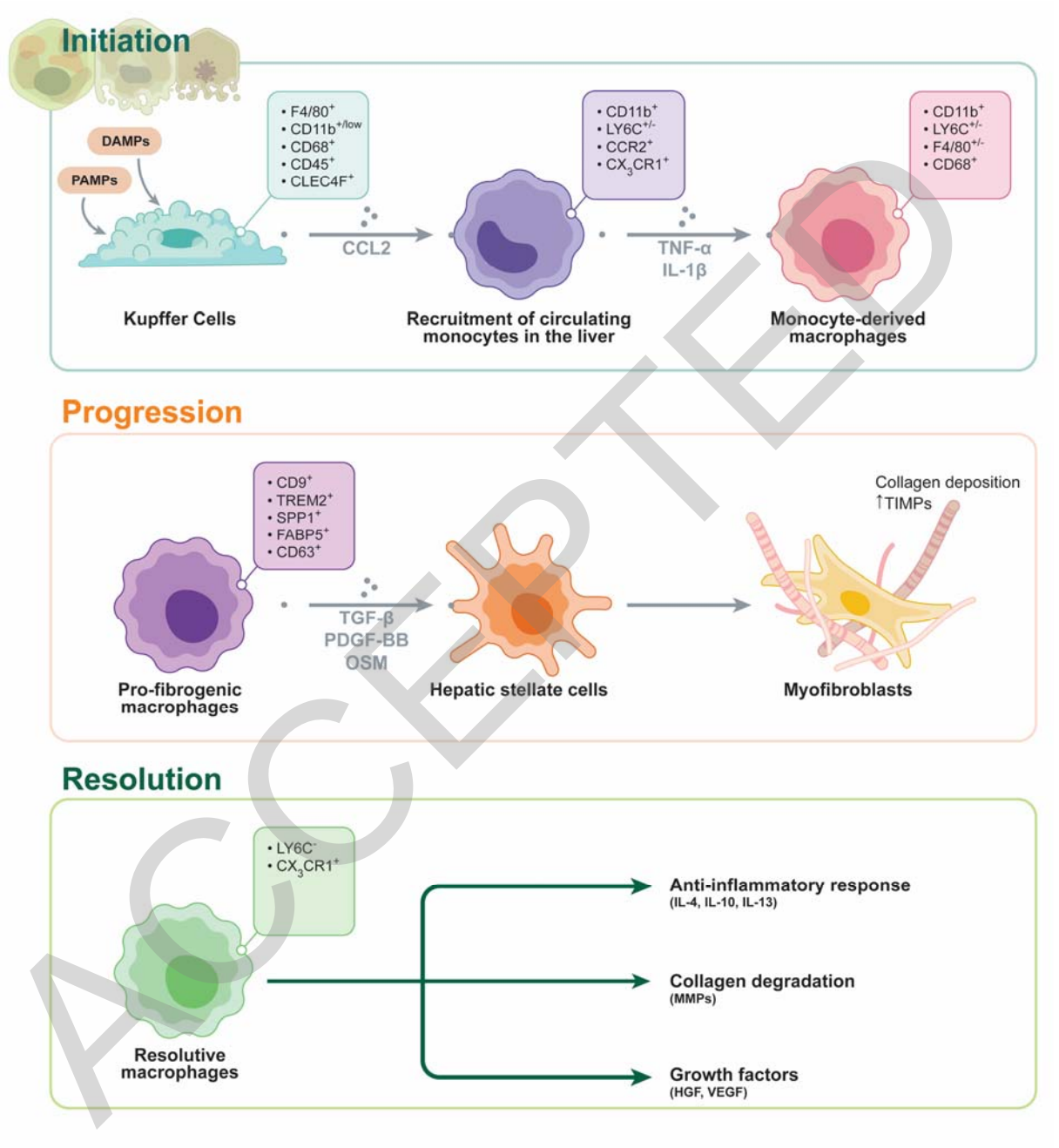

Figure 1. Hepatic macrophage populations during the initiation, progression and resolution of liver injury. Upon liver injury caused by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), the recruited MDMs promote pro-inflammatory immune response. During the progression of liver injury, MDMs support fibrogenesis through the secretion of pro-fibrogenic factors that activate hepatic stellate cells.

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Macrophages also play essential roles in the resolution of liver inflammation and fibrosis, promoting an anti-inflammatory response and secreting ECM remodeling metalloproteinases and/or growth factors. Abbreviations: CCL2, C-C motif chemokine ligand 2; DAMPs, damage-associated molecular patterns; HGF, hepatocyte growth factor; IL, interleukin; IL-1β, interleukin 1 beta; MMPs, matrix metalloproteinases; OSM, oncostatin M; PAMPs, pathogen-associated molecular patterns; PDGF-BB, platelet-derived growth factor-BB; TGF-β, transforming growth factor beta; TIMPs, tissue inhibitors of metalloproteinases; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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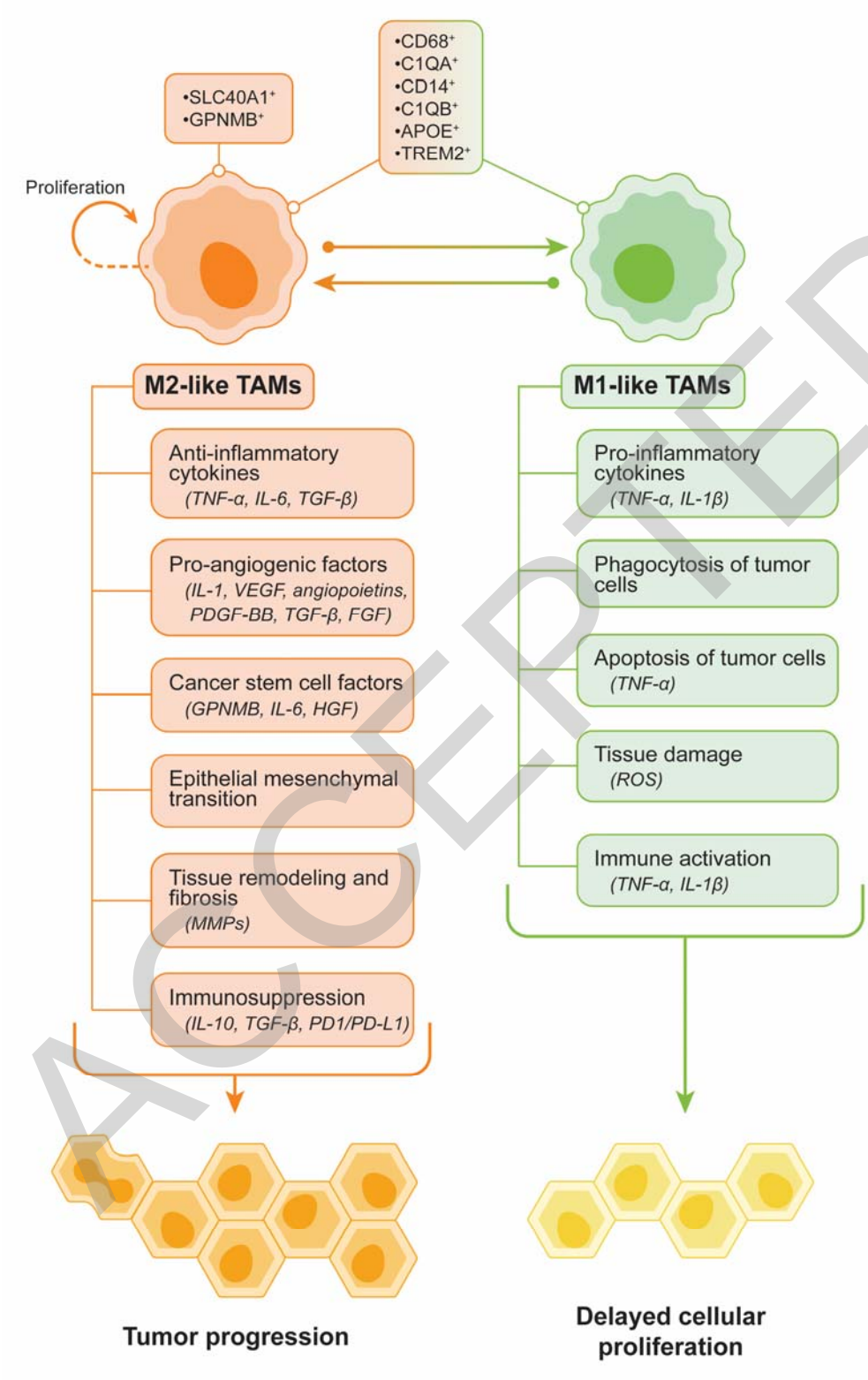

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Figure 2. Anti-tumoral and pro-tumoral functions of tumor-associated macrophages (TAMs). Macrophage plasticity allows the switch between M1-like and M2-like macrophages.

In the tumor microenvironment, several molecules maintain TAMs phenotypes and functions. During tumor progression, M2-like macrophages with pro-tumoral functions are conditioned by immuno-suppressive mediators. M2-like-TAMs secrete anti-inflammatory cytokines and molecules that promote angiogenesis, tumor proliferation, epithelial mesenchymal-transition, and continuous matrix remodeling. In addition, several immuno-suppressive molecules are produced that support regulatory T cells. M1-like macrophages with anti-tumoral functions stimulate tumor resolution and slow down tumor progression. M1-like-TAMs produce pro-inflammatory cytokines and actively phagocytose tumor cells and release TNF-α and ROS to directly activate apoptosis in cancer cells. Abbreviations: FGF, fibroblast growth factor; GPNMB, glycoprotein NMB; HGF, hepatocyte growth factor; IL, interleukin; IL-1β, interleukin 1 beta; MMPs, matrix metalloproteinases; OSM, oncostatin M; PDGF-BB, platelet-derived growth factor-BB; PD1, programmed death 1; PD-L1, programmed death ligand 1; ROS, reactive oxygen species; TAMs, tumor-associated macrophages; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.

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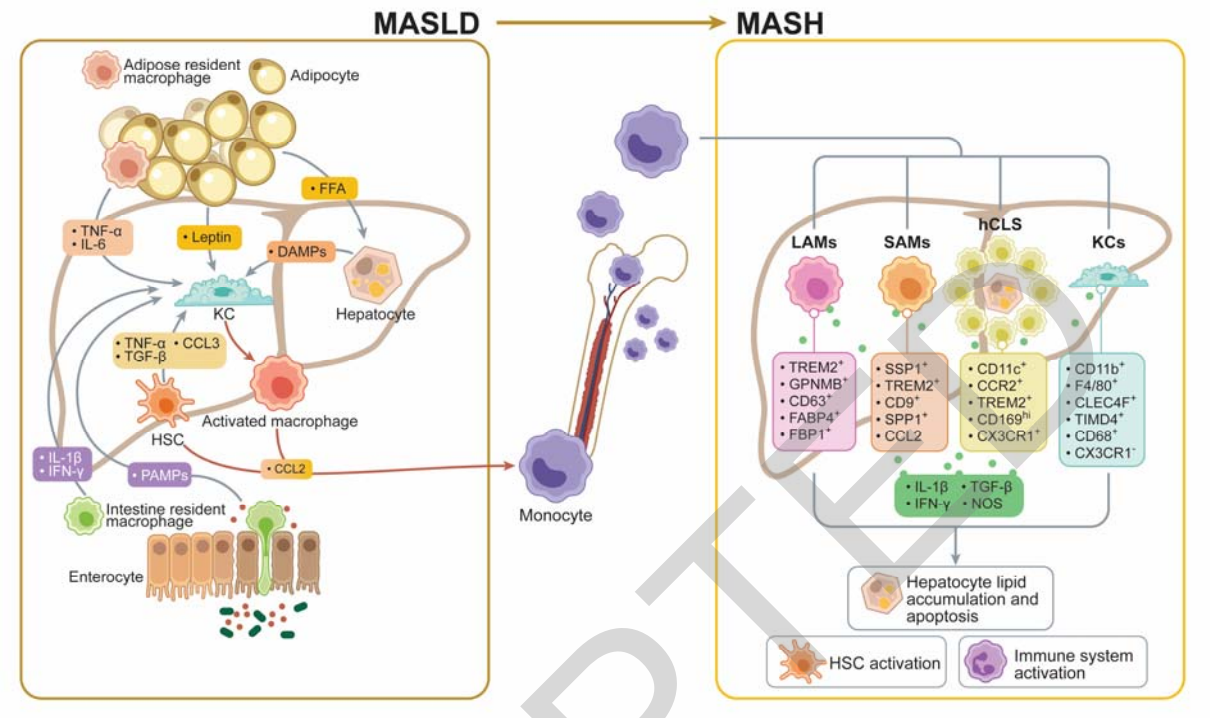

Figure 3. Molecular signals from macrophage activation involved in the progression from metabolic dysfunction-associated steatotic liver disease (MASLD) to metabolic dysfunction-associated steatohepatitis (MASH). Extrahepatic organs involved in metabolic syndrome contribute to MASLD aggravation. Metabolic dysfunction of adipose tissue induces resident macrophage activation, generating adipose tissue inflammation and activation of liver KCs because of cytokine release. Inflamed adipose tissue secretes leptin and FFA into the bloodstream directly activating KCs and loading hepatocytes with FFA. Intestine barrier permeabilization allows PAMPs influx to the liver activating KCs. Intestine resident macrophages are also activated by PAMPs and secrete cytokines to the bloodstream that also activate KCs. In the liver, hepatocytes loaded with FFA secrete DAMPs and HSCs secrete cytokines, which stimulate KCs. Activated KCs and HSCs secrete CCL2, which promote monocyte recruitment from the bone marrow. Recruited monocytes infiltrate into the liver and differentiate into LAMs, SAMs or hCLS, which together with activated KCs secrete cytokines. This cytokine storm causes HSC activation, hepatocyte apoptosis and immune system activation culminating in MASH progression. Abbreviations: CCL, C-C motif chemokine ligand; DAMPs, damage-associated molecular patterns; FFA, free fatty acids; hCLS, hepatic crown-like structures; HSCs, hepatic stellate cells; IL-1β, interleukin 1 beta; IL-6, interleukin 6; INF-γ,

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interferon gamma; KCs, Kupffer cells; LAMs, lipid associated macrophages; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; NOS, nitric oxide synthase; PAMPs, pathogen-associated molecular patterns; SAMs, scar associated macrophages; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha.

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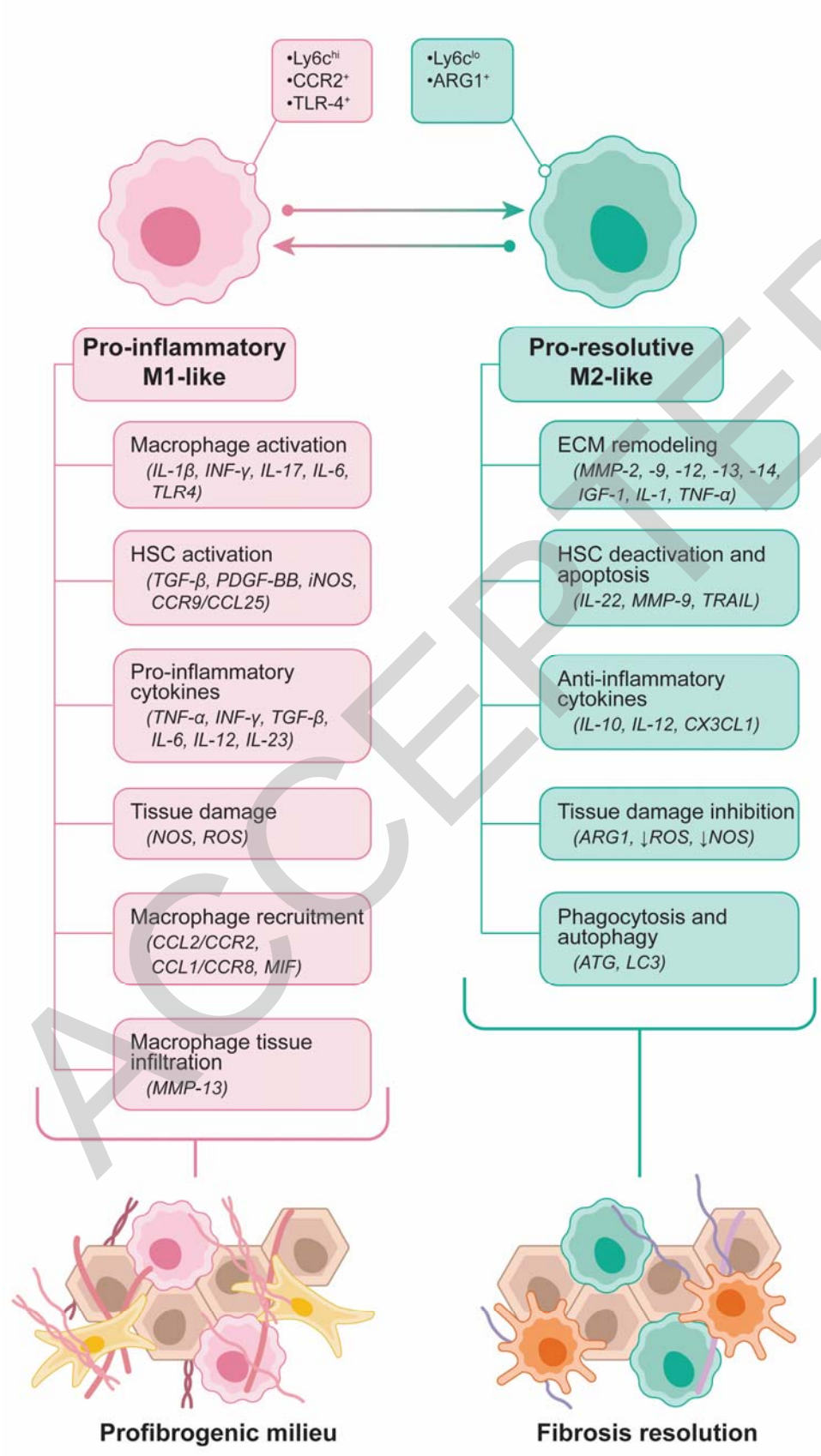
•Ly6c^hi
•CCR2+
•TLR-4+
•Ly6c^lo
•ARG1+
Pro-inflammatory M1-like
Macrophage activation (IL-1β, INF-γ, IL-17, IL-6, TLR4)
HSC activation (TGF-β, PDGF-BB, iNOS, CCR9/CCL25)
Pro-inflammatory cytokines (TNF-α, INF-γ, TGF-β, IL-6, IL-12, IL-23)
Tissue damage (NOS, ROS)
Macrophage recruitment (CCL2/CCR2, CCL1/CCR8, MIF)
Macrophage tissue infiltration (MMP-13)
Pro-resolutive M2-like
ECM remodeling (MMP-2, -9, -12, -13, -14, IGF-1, IL-1, TNF-α)
HSC deactivation and apoptosis (IL-22, MMP-9, TRAIL)
Anti-inflammatory cytokines (IL-10, IL-12, CX3CL1)
Tissue damage inhibition (ARG1, ↓ROS, ↓NOS)
Phagocytosis and autophagy (ATG, LC3)
Profibrogenic milieu
Fibrosis resolution

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Figure 4. Pro-inflammatory and pro-resolutive functions of macrophages during liver fibrosis. Successful fibrosis resolution relies on macrophage plasticity and a phenotypical switch from pro-inflammatory or M1-like phenotype to a pro-resolutive or M2-like phenotype. Pro-inflammatory macrophages are crucial for immune system activation through cytokine secretion and for the clearance of cellular debris. Although M1-like phenotype is required for the initial pro-inflammatory response, sustained M1-like polarization contributes to chronic inflammation and fibrosis. On the other hand, pro-resolutive macrophages play a key role in fibrosis resolution since they ameliorate tissue inflammation, tissue damage and fibrogenesis in liver tissue. M2-like macrophage phenotype deactivate HSCs, induce apoptosis, and engulf apoptotic cells and extracellular matrix. Abbreviations: Abbreviations: ARG1, arginase 1; ATG, autophagy protein; CCL, C-C motif chemokine ligand; CCR, C-C chemokine receptor; CX3CL1, C-X3-C motif chemokine ligand 1; HSCs, hepatic stellate cells; IGF-1; insulin-like growth factor 1; IL-1β, interleukin 1 beta; IL, interleukin; INF-γ, interferon gamma; iNOS, inducible nitric oxide synthase; LC3, microtubule-associated protein 1A/1B-light chain 3; MIF; macrophage migration inhibitory factor; MMPs, metalloproteinases; NOS, nitric oxide synthase; PDGF-BB, platelet-derived growth factor-BB; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha; TRAIL, TNF-related apoptosis-inducing ligand.

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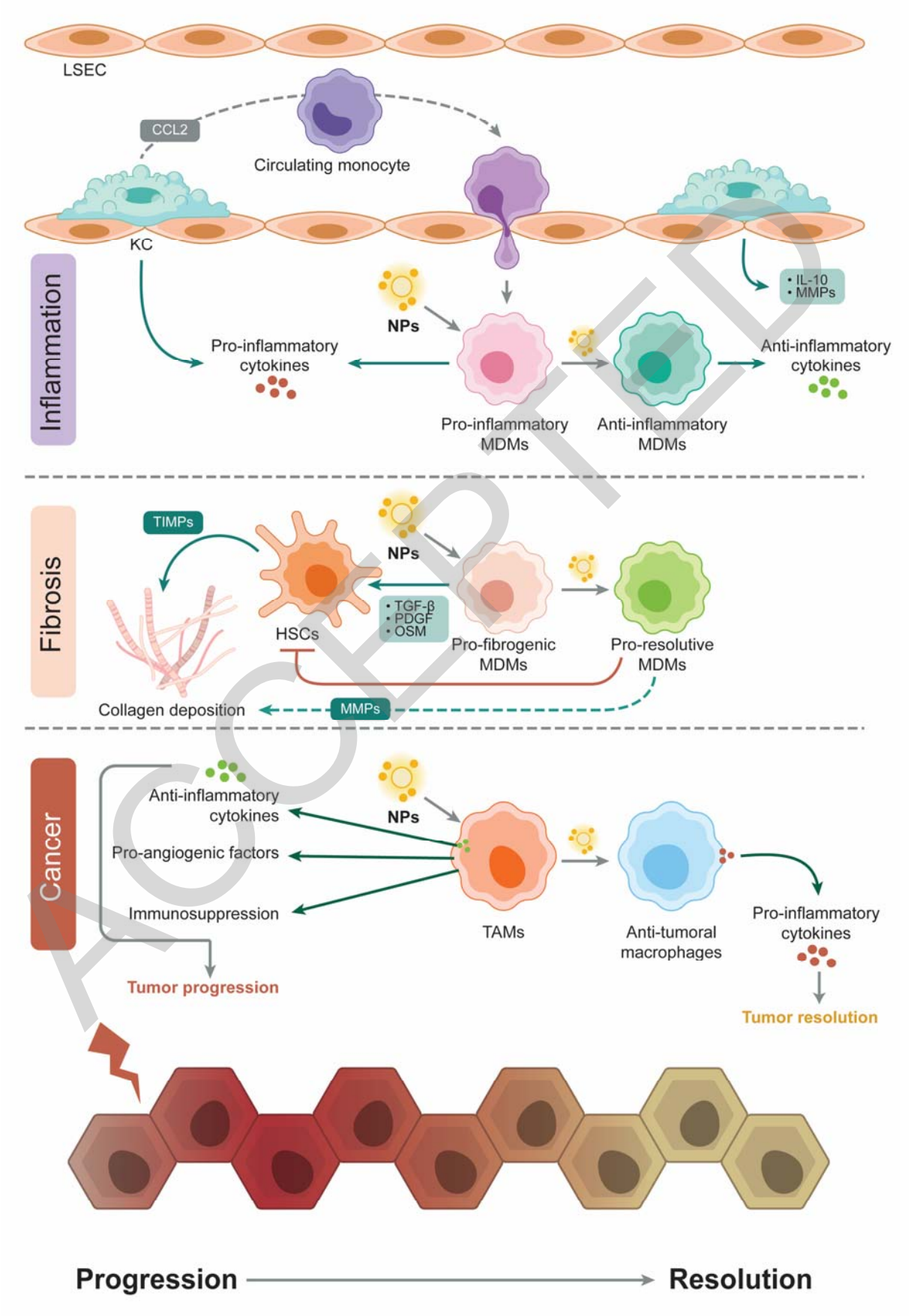

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Figure 5. Nanoparticles to target and reprogram macrophages in liver diseases. Novel nanomedicines involving therapeutic nanoparticles may modulate macrophage polarization from inflammatory, pro-fibrogenic, and pro-tumoral (TAMs) phenotypes to anti-inflammatory, pro-resolving, and anti-tumoral phenotypes. Abbreviations: CCL2, C-C motif chemokine ligand 2; HSC, hepatic stellate cell; IL-10, interleukin-10; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; MDMs, monocyte-derived macrophages; MMPs, matrix metalloproteinases; NPs, nanoparticles; OSM, oncostatin M; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor beta; TIMPs, tissue inhibitors of matrix metalloproteinases.

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Table 1. Macrophage subsets in healthy and diseased liver.

Macrophage subtype	***Markers***		***Functions***	***References***
	Mouse	**Human**		
Kupffer cells	$CD11b^+$ $F4/80^+$ $CD68^+$ $CD45^+$ $CLEC4F^+$ $TIMD4^+$ $CX3CR1^-$	$CD14^+$ $CD16^+$ $CD68^+$ $CD163^+$ $MARCO^+$ $CD5L^+$ $TIMD4^+$	Phagocytosis Regulation of iron and lipid metabolism Immunological tolerance	(13), (14), (15), (25)
Monocyte-derived macrophages	$CD11b^+$ $F4/80^{+/-}$ $LY6C^{+/-}$ $CSF1R^+$ $CCR2^+$ $CX3CR1^+$ $CD64^+$	$CD11b^+$ $CX3CR1^+$ $CCR2^+$ $CD14^+$ $CD16^{+/-}$	Innate immune response to injury Inflammatory, angiogenic and fibrogenic activity Immuno-suppressive functions	(1), (13), (14), (15), (25)
Inflammatory macrophages	$LY6C^+$ $iNOS^+$ $TNF\text{-}\alpha^+$ $IL\text{-}1\beta^+$ $CCL2^+$ $CCR2^{+/-}$	$CD14^+$ $CD16^+$	Antigen presentation Th1 immunological activation Pathogen elimination Antitumor activity	(1), (5), (25), (26)
Lipid-associated macrophages	$TREM2^+$ $CLEC4F^-$ $TIM4^-$ $GPNMB^+$ $CD63^+$ $CD9^+$ $FABP4^+$ $FBP1^+$	$TREM2^+$ $CD9^+$ $LIPA^+$ $OTOA^+$ $FBP1^+$ $GPNMB^+$	Regulation of liver lipid metabolism Removal of dying hepatocytes Homeostasis of bile ducts in healthy livers Pro-inflammatory damage in MASLD Anti-inflammatory repair during MASH	(17), (18), (27), (28), (29), (30)

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Scar-associated macrophages	SPP1^{+} TREM2^{+} CD9^{+} MNDA^{+} CCL2^{+} CXCL8^{+} LGALS3^{+} TNFSF12^{+}	TREM2^{+} CD9^{+} SPP1^{+} CD63^{+} LGALS3^{+}	Activate HSC	(14), (30), (31)
			Interaction with fibrotic niche	
			Th1 cell activation	
			Modulate immune system activation	
Hepatic Crown-Like Structures	CD11c^{+} CCR2^{+} TREM2^{+} CD169^{hi} F4/80^{hi} CX3CR1^{+}	CCR2^{+} CD68^{+}	Hallmark MASH progression.	(27), (30), (32)
			Encircling dead hepatocytes	
			Activate HSCs	
			Surrounding lipid and fibrosis areas	
Resolutive macrophages	LY6C$^{+/-}$ CSF1R^{+} CX3CR1^{+} MMP9^{+} MMP12^{+}	CD16^{+} CD163^{+}	Pro-inflammatory	
			Anti-inflammatory activity	(1), (25), (33), (34), (35)
			Wound healing	
			Th2 immune response	
			Fibrosis resolution	
			Phagocytosis of apoptotic cells	
			Regulation of HSCs	
			Lipid metabolism	
			Immunosuppression	
Tumor-associated macrophages	CD11b^{+} F4/80^{+}	CD68^{+} CD14^{+} APOE^{+} C1QA^{+} C1QB^{+} TREM2^{+}	Tissue remodeling	
			Pro-tumoral activity	
			Formation of the tumor microenvironment	(1), (25), (36), (37), (38)
			Tumor growth and metastasis	

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ECM remodeling / fibrosis

Angiogenesis

Immunosuppression

ACCEPTED

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Table 2. Pre-clinical studies and clinical trials for the treatment of metabolic dysfunction-associated steatotic liver disease (MASLD), metabolic dysfunction-associated steatohepatitis (MASH) and fibrosis.

	PRE-CLINICAL STUDIES	CLINICAL TRIALS
NUCLEIC ACIDS	**pRNF41-DGNPs (33):** Mice CCl_4 and TAA fibrosis and hepatectomy model. *Nanoparticles treatment reduced liver inflammation and fibrosis, and induced hepatic regeneration.*	
	mLNP-siHMGB1 (92): Mannose-modified HMGB1-siRNA loaded lipid particle. MASH model mice. *Nanoparticles treatment reduced hepatic inflammation and steatosis.*	
	STAT3 and HIF-1α siRNA-loaded CL4H6-LNPs (93): Human tumor xenograft model in nude mice. *Nanoparticles treatment resulted in an increase of infiltrated macrophages and M1 macrophages, and a reversion of pro-tumorous TAMs functions.*	
DRUGS	**Annexin A5 (79):** High fat diet-induced MASH mice. *Improves steatosis, inflammation and fibrosis.*	**Resmetirom (83):** Approved by FDA. *Reestablished RGS5 expression that regulates inflammatory response in MASH.*
	Celastrol (80): High fat diet-induced MASLD mice. *Alleviates lipid accumulation, inflammation and fibrosis.*	**Cenicriviroc (96):** Phase III clinical trial in patients with MASH. *CCR2/CCR5 antagonist.*
	Rosiglitazone (47): MASLD treated mice. *PPAR-γ regulator.*	**Elafibranorn (97):** Phase IIb clinical trial in patients with MASH. *PPAR-α and -γ agonist.*
	19,20-EpDPE (52): Fat-1 treated mice.	**Selonsertib (98):**

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OTHERS

Attenuates hCLS formation by GPR120 binding.

NaA (81):
MCD diet mouse model.
Decrease macrophage infiltration reducing TNF-α and IL-6 expression.

Tim3 Activation (84):
MCD diet mouse model.
Reduces ROS formation.

Anti-CD163-IgG-dexamethasone conjugate (94):
Diet induced MASH in rats.
Decrease inflammation.

PPARγ agonist GW1929-DGNS (95):
Mice CCl_4-fibrosis model.
Nanoparticles treatment reduced hepatic inflammation and fibrosis.

Ac-6-FP and anti-MR1 antibodies (100):
Mice MCD diet and CCl_4-fibrosis model.
Downregulates CCL2/CCR2 axis.

Liposome administration (101):
Mice CCl_4-fibrosis model.
Phenotypical shift towards pro-resolutive macrophages.

BMDM delivery (102):
Mice CCl_4-fibrosis model.
Upregulates $MMP13^+$ macrophages.

CAR-M targeting uPAR (103):
Mice CCl_4 and (DDC) diet feeding fibrosis models. Mice high fat, cholesterol, and fructose (HFCF) diet-induced non-alcoholic steatohepatitis model.
CAR-M reduces fibrosis, restores liver function, and activates specific T-cell response.

Phase III clinical trial in patients with fibrosis.
ASK1 inhibitor.

Lanifibranor (99):
Phase IIb clinical trial in patients with fibrosis.
Pan-PPAR agonist, anti-inflammatory polarization of macrophages.

Autologous macrophage delivery (104):
Phase I clinical trial in patients with fibrosis.
Safety evaluation.